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YUN-TAO SUN

COLLEGE OF MEDICINE, NATIONAL TAIWAN UNIVERSITY

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A STUDY OF ANTIGENIC ACTIVITY OF SOME PLANT TOXALBUMINS

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A STUDY OF ANTIGENIC ACTIVITY OF SOME PLANT TOXALBMINS

Some plant toxalbumins, such as ricin from Ricinus Communis (Euphorbiaceae) and abrin from Abrus precatorius (Leguminosae) are extremely toxic. Ricin, the highly toxic, hemagglutining protein is of great interest in medicine. For such a toxalbumin when injected in small doses may act as an antigen and produce in the body an antitoxin analogue to that produced against bacteria or venom. The isolation of ricin was first realized by Stillmark[1]. Earlier methods of preparation were carried out before the development of modern physiochemical and immunochemical methods for characterizing proteins and data are therefore laking on the purity and homogeneity of the products obtained. In recent years, the different toxic fractions of ricin have been prepared by Kabat, Heidelberger and Bezer[2] by fractional precipitation with sodium sulfate (1947). Kunitz and McDonald[3] have obtained the most toxic fraction by first precipitation with Sodium Sulfate and then adjusted the iso-electric point of toxalbumin Solution at pH 5.2-5.5 (1948). Moulé[4] stated that the highly toxic fraction of ricin was prepared by precipitation with half saturation of ammonium sulfate (1951). The isolated fractions of ricin prepared by previous authors were not pure and homogenous substances.

In the present report a summary is given of a portion of a study on the extraction and purification of ricin. The nature, toxicity and antigenic activity of ricin are also Studied.

EXPERIMENTAL

Extraction and Purification of Ricin

1,380 Gm. of castor bean were ground and macerated with 1,400 ml. of ether at room temperature for 12 hours. Pressed out the solvent. Repeated the maceration as the previous time. The castor bean powder was then air dried. The castor meal was extracted twice with 1.5 L. of alcohol at room temperature. Removed the alcohol by pressing and dried the bean powder under reduced pressure. The defatted matter, weighing 410 Gm., was then macerated twice with 2 L. of 10% sodium chloride solution at 3-4°C for 24 hours. By filtraction, the filtrate was freed from sodium chloride by dialysis. The non-toxic globulin precipitated was centrifuged off. The proteins of the supernatant liquid were precipitated by saturation with ammonium sulfate, and after 15 hours at about 10°C, centrifuged the mixture. The precipitate was dissolved in water and freed of the insoluble matter by centrifuging. Repeat the precipitation and dissolution.

To the clear liquid measured 90 ml., 45 ml. of saturated ammonium sulfate Solution was added (1/3 saturation) and kept the mixture at 3-4°C for 24 hours. The forming precipitate was centrifuged off. To the liquid of a volume of 130 ml., 44 ml. more of saturated ammonium sulfate solution was then added (half saturation) and kept the mixture at about 10°C for 24 hours. The precipitate separated from centrifuging, was dissolved in a small amount of water (the supernatant liquid from centrifuging was reserved for further treatment). Repeated the precipitation and dissolution and finally the solution was dialysed. The clear solution was freeze-dried in a Stoke's freeze-dryer, model 2003 F2 (equipment with freon as freezing agent, drying under high vacuum at 300 u over 16 hours). The white porous powder, Ricin fraction I (Ricin I), 904 mg. was obtained (yield: 0.066%).

The above reserved supernatant liquid from half saturation of ammonium sulfate was saturated again with ammonium sulfate. Kept the mixture at 10°C for 24 hours. Collect the precipitate after centrifuging. The precipitate was dissolved in a small amount of water and made the solution salt free by dialysis. The liquid was freeze-dried in a Stoke's freeze-dryer, giving 2.981 Gm. of a white, porous powder, Ricin fraction II (Ricin II), (yield: 0.216%).

Properties of Ricin Fractions

Determination of the sedimentation constant of ricin fractions was carried out by using 1% solution of ricin fractions in 0.2 M sodium phosphate in an ultracentrifuge. The data of determination were as follows:

		Molecular weight calculated
Ricin I	$S_{20} = 4.78 \times 10^{-13}$	75,000
Ricin II	$S_{20} = 5.30 \times 10^{-13}$	85,000

Ricin II was found to have a molecular weight of about 85,000. The sedimentation constant of Ricin I was slightly lower, leading to a molecular weight of 75,000, a value not considered significantly different from that of Ricin II, since the precise temperature control was not possible during the measurement of sedimentation.

Ricin I showed a lower optical rotation (-28°) than did the Ricin II (-32°).

Table I Properties of Ricin Fractions

	Ricin I	Ricin II
$(\alpha)_D$ degrees	-28°	-32°
Molecular weight	75,000	85,000

The Ricin I and II were determined qualitalively by paper chromatography. A descending method, on Whatman paper No. 1 and a solvent of citrate buffer with pH 6.0 (0.75 ml. of 0.02 M sodium citrate, 9.25 ml. of 2 N HCL, and 50 Gm. of sodium chloride per liter) were employed. The temperature was kept at 20°C. Using ninhydrin as the spraying agent, it showed that Ricin I gave two nearby spots, and Ricin II, three spots.

Toxicity Test of Ricin Fractions

The toxicity test of ricin fractions was determined by intraperitoneal injection of 0.5 ml. of the ricin solution in serial concentrations. Three mice weighing 20±3 gm. were used as a lot, being injected with each concentration of the ricin solution. Death or survival for four days was used as the end point. A dose of 1 mcg. of Ricin I had no significant toxic effect on mice weighing 20 Gm. when perorally administered but was lethal when administered intraperitoneally.

Table II Toxicity Test of Ricin Fractions

Quantity of ricin fractions injected (mcg.)	Intraperitoneal toxicity for mice	
	Ricin I	Ricin II
0.5	0/3	
1.0	3/3	
1.5	3/3<60hr.	
5.0		3/3
5.5		3/3 96hr.

Enzymatic Hydrolysis of Ricin Fractions

The digestion of Ricin I by pepsin at pH 4 or by trypsin at 7.4 was found to take place slowly. Action for 3 days, the digested ricin gave a slight decline in its toxicity. 1 mcg. dose of the digested Ricin I by intraperitoneal injection caused death of the mice in 72 - 96 hours. When a prolonged enzymatic digestion was made for 2 weeks, it broke down about 39 - 48% of the low molecular products.

Table III Two-weeks Enzymatic Digestion of Ricin I

	Control	Pepsin digest	Control	Trypsin digest
Precipitable at by Trichloroacetic acid, %				
Trichloroacetic acid, %	100	52		
Half saturation with ammonium sulfate, %	100	52	100	61

Immuno-chemical Properties of Ricin Fractions

The antitoxic serum to Ricin I was prepared by immunization of rabbits with a formalinized toxoid prepared as follows: A solution containing 5 mg. of Ricin I per ml. buffered at 7.4 with 0.02 M sodium phosphate and 0.15 M sodium chloride and with 0.5% formalin was kept at 37°C for 5 days (in some instances 5% formalin was used). This procedure resulted in about a 100 to a 1000 fold reduction in toxicity when 0.5% formalin was used, and about 1000-fold reduction in toxicity with 5% formalin. Marked loss of antigenicity occured with formalin at pH 8.5 and above.

Because of the decrease of toxicity of the ricin toxoid and the extreme susceptibility of the rabbit to ricin, it was found necessary to give each rabbit subcutaneous injections of 25, 50 and 50 mcg. of toxoid at 5 day intervals to induce some immunity before intravenous injections were started. Each rabbit then received 2-4 intravenous injections weekly for 4 weeks, as follows: two injections of 0.1 mg., two of 0.3 mg., four of 0.5 mg., four of 1.5 mg., and four of 5.0 mg. of ricin toxoid. The animals became so resistant to the toxic effects of ricin that immunization could be continued with equal doses of an alum precipitated undetoxified ricin. Rabbits were bled 5 days after the injection. The effect of antitoxic serum has been showed by neutralizing the toxic effect of ricin on intraperitoneal injection into mice.

DISCUSSION

The defatted castor meal firstly extracted with 10% sodium chloride solution, followed by fractional precipitation with ammonium sulfate (1/3, 1/2 and full saturation), the highly toxic ricin fractions I and II were obtained. The qualitative determination of ricin fractions by paper chromatographic method showed that Ricin I gave two nearby spots, and Ricin II, three. It seems that Ricin I and II are still not pure, homogenous substances, same as those ricin fractions prepared by Kabat, Heidelberger and Bezer, Kunitz and McDonald as well as Moulé etc.. An attempt was made for the purification of Ricin I & II by using ion-exchange resin column chromatography. No favorable results obtained.

Ricin I & II are different by their molecular weight of 75,000-85,000. Ricin I showed a lower rotation (-28°) than did the Ricin II (-32°). As to the toxicity of ricin, 1 mcg. dose of Ricin I killed a mouse (20 Gm.) by intraperitoneal injection after an interval of 4 days, while in the case of Ricin II, a dose of 5 mg. gave the same lethal effect. It is interest to notice that a dose of 1 mcg. of Ricin I had no significant toxic effect on mice weighing 20 Gm. when perorally administered but was lethal when administered intraperitoneally. It seems that the ricin undergo hydrolyse and lose part of its toxicity in the gastro-intestinal tract of mouse.

In the enzymatic hydrolysis of ricin fractions, the digestion of Ricin I by pepsin at pH 4 or by trypsin at 7.4 was found to take place slowly. Action for 3 days, the digested Ricin I gave a slight decline in its toxicity. It has been showed that ricin was somewhat resistant to the proteolytic enzymes. It is somewhat contradictory to the phenomenum that the ricin I had no significant toxic effect in mouse when administered perorally. A prolonged enzymatic digestion for 2 weeks, it broke down about 39 - 48% of the low molecular products.

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